Biochemical Properties of Pork Muscle in Relation to Curing

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SUMMARY

With differential absorption manometry it was shown that a gas having the solubility properties of nitric oxide is produced when pork muscle mince is incubated with sodium nitrite, and the presence of nitric oxide in the gaseous products has been directly confirmed. Evidence is presented to show that the production of presumptive nitric oxide was associated with mammalian rather than bacterial enzyme systems. No similar activity was observed toward nitrate, but the reduction of metmyoglobin on incubation with pork muscle mince has also been demonstrated. Purified pig heart metmyoglobin was shown to combine directly with gaseous nitric oxide, giving a complex of absorption spectrum similar to that of nitrosylmyoglobin, but with absorption maxima displaced toward the blue end of the spectrum. This complex decomposed rapidly on contact with air, giving a brown product showing no characteristic absorption bands.

INTRODUCTION

The characteristic color developed during the curing of meat is due to nitrosylmyoglobin, formed by the combining of nitric oxide with the reduced form of the meat pigment (Haldane, 1901.) Satisfactory color development involves the prior formation of both reactants, the reduced pigment by conversion of the oxy- and metforms and the nitric oxide by reduction of nitrite, either introduced as such during curing or itself formed by reduction of nitrate introduced as saltpeter. The necessity for reducing conditions was demonstrated by Brooks (1937), who showed that sodium nitrite reacted with hemoglobin to yield nitrosylhemoglobin only in the presence of a reducing agent such as dithionite; in the absence of a reducing agent, equimolecular proportions of nitrosylhemoglobin and methemoglobin were produced, the brown color of the latter predominating in the mixture.

The nature and sources of the reducing systems operative during curing have not been clearly established. Bacterial reduction of both nitrate and nitrite is known (Eddy, 1958), and chemical reducing agents (ascorbic and erythorbic acids) are used in commercial curing as processing aids. The persistence of respiratory enzyme systems is shown by the depletion of oxygen in blocks of muscle tissue, and studies by Andrews *et al.* (1952) and Grant (1955) have shown succinic dehydrogenase to be among the most stable of these systems. The activity of this system in bovine tissue, as shown by the post-mortem oxygen requirements, has been studied by Urbin and Wilson (1961). The present work was done to determine whether surviving enzyme systems in pork muscle were capable of effecting the specific reductions essential for color formation. The work entailed the preparation of purified myoglobin from pig hearts; the pigment was isolated in the form of metmyoglobin, and in this form it combined directly with nitric oxide to form a nitric oxide-metmyoglobin complex. Some of the properties of this complex are also reported here.

EXPERIMENTAL METHODS

Reduction of nitrite. Reduction of nitrite was studied by gas-exchange measurements in a conventional Warburg apparatus at 37°C., using 3 g of muscle tissue from pork hocks 18–24 hr after slaughter. The meat was minced through a 2-mm plate and supported in 3.0 ml of 0.2*M* phosphate buffer, pH 6.0, containing 10.0 mg/100 g of chloromycetin. Anaerobic conditions were obtained by flushing the apparatus thoroughly with argon. One-half ml of 3.0% (w/v) sodium nitrite was added to the meat by tipping in from the side-arm after equilibration in the usual manner. Observations were made by differential absorption, using 5% potassium hydroxide to absorb carbon dioxide, and alkaline sodium sulfite solution (Treadwell and Hall, 1942) to absorb both carbon dioxide and nitric oxide. All experiments involved replicate flasks, usually three.

The gaseous products were examined by mass spectrometry and by infrared absorption spectrophotometry. For production of gas for these examinations, the reaction was allowed to proceed for 2 hr at 37°C under generally similar conditions. The gaseous products were transferred directly to the mass spectrometer; for infrared absorption measurements, transference was made over phosphorus pentoxide into an evacuated gas cell (rock-salt plates; path length 6 cm) through glass tubing previously flushed with argon.

Reduction of nitrate. Conditions were exactly as described for nitrite except that the side-arm of the Warburg apparatus contained 0.5 ml of 3.0 (w/v) potassium nitrate. Secondary additions of sodium nitrite solution (previously bubbled with argon to remove dissolved oxygen) to Warburg flasks were made through the gas ports into the side-arms by suction resulting from temporary removal of the flasks and manometers to room temperature from the constant-temperature bath at 37°C.

Reduction of metmyoglobin. Reduction of metmyoglobin was followed spectrophotometrically. Satisfactory clarification for this purpose without loss of color was not found possible either by centrifugation at 3000 rpm or by filtration (with or without filter aids or pre-treatment with basic lead acetate) after incubation of muscle mince with metmyoglobin in 0.2*M* phosphate buffer of pH 6.0. Omission of the phosphate buffer and addition of ethanol or acetone to 20% v/v after incubation gave satisfactory clarification by centrifuging 10 min at 3000 rpm, although inexplicable failure to achieve clarification was observed on a few occasions. Acetone appeared to give the most consistent results, and was adopted for use. In spite of omission of the buffer, pH values of the mince and water slurries were maintained close to 6.0 by the meat alone. In carrying out the tests, 2 g of pork muscle mince was incubated for 2 hr with metmyoglobin (8.0 mg) in aqueous solution (6.0 ml) containing 6.7 mg/100 g of chloromycetin. Incubations were carried out in Thunberg tubes at 37°C under argon at reduced pressure. After incubation for 2 hr, sodium iodoacetate solution was added to 0.011*M*. Controls were treated with a similar addition of sodium iodoacetate

before the introduction of the metmyoglobin. The incubation products were bubbled with oxygen for approximately 1 min, acetone was added to give 20% (v/v), and the solutions were centrifuged 10 min at 3000 rpm and filtered through Whatman No. 1 filter paper; the final filtrate was normally clear and bright. The composition of the pigment mixture was obtained from the ratio of the optical densities at 577 and 593 mμ, the choice of wavelengths being based on the observed properties of pig heart oxy- and metmyoglobin. For determination of total pigment, the optical density at 525 mμ, which is isosbestic to reduced, oxy-, and metmyoglobin at a point where all three absorb well, was used. Each determination was carried out in quadruplicate; variation of individual determinations from the mean was rarely greater than 10%.

Bacterial counts. Counts were made on the residue from the Warburg incubation. After aseptic removal of the absorption liquor the meat mixture was disintegrated in sterile saline, and decimal dilutions were plated on 'Oxoid' plate-count agar (0.5% tryptone, 0.24% yeast extract, 0.1% dextrose, and 1.5% agar at pH 7.0); the plates were incubated 24 hr at 37°C before counting.

Preliminary experiments with a pure culture of *Escherichia coli* indicated that chloromycetin in concentration at least ten times as great as that resulting from dilution of the incubation products had no appreciable effect upon plate counts.

Preparation of pig heart metmyoglobin. In an adaptation of the method of Theorell (1932), fresh washed pig hearts (24 lb) were minced and extracted twice with 4.5 L of cold distilled water. The combined extracts, separated by filtration under pressure in a tincture press, were treated with a slight excess of basic lead acetate and centrifuged. Solid ammonium sulfate was added, with stirring, to a concentration of 38% (w/v), and the brown precipitate that separated after 2 days at 0°C was filtered off and discarded. The concentration of ammonium sulfate was raised to 46% (w/v); the dark-brown precipitate that separated after 16 hr at 0°C was filtered off, redissolved in water, and dialyzed against running tap water in a Visking cellulose casing. Final dialysis overnight against distilled water was followed by freeze drying. The yield of brown feathery powder was 6.2 g.

Preparation of nitric oxide. Fifty percent (v/v) sulfuric acid was dripped onto a mixture of potassium iodide (8 g) and sodium nitrite (16 g) in 50 ml water. The gaseous product was washed by passage through concentrated sulfuric acid and 10% potassium hydroxide.

RESULTS AND DISCUSSION

Reduction of nitrite. The differentiation effected by the use of selective absorbents is illustrated in Fig. 1, which shows a typical set of observations at 37°C. In this experiment, nitrite was added to the muscle mince at the beginning of the experiment, as soon as the preliminary equilibration was complete. It will be seen that, even with the alkaline sulfite absorbent, evolution of non-absorbable gas was appreciable. The nature of this gas has not been established, but it seems likely that it may be essentially nitrogen, produced during the incubation period by the normal van Slyke reaction. The differential between the alkaline sulfite and the alkaline absorbents represents gas soluble in alkaline sulfite but not in alkali, i.e., gas having the solubility properties of nitric oxide, and referred to for convenience as "presumptive nitric oxide." Finally, the difference between the alkali and the water absorbents represents carbon dioxide production.

Fig. 2 illustrates the effect of the introduction of nitrite to a muscle mince previously incubated for 30 min under anaerobic conditions. The immediate stimulus in total gas production is evident, and the differential absorption indicates the evolution of presumptive nitric oxide as well as of nitrogen or other gas insoluble in the alkaline sulfite reagent. Evolution of carbon dioxide was maintained. In some experiments a slight over-all gas absorption in the alkaline reagents was observed in the early stages of the incubation; a typical experiment of this type is shown in Fig. 3. It was thought that this initial absorption might be due to residual traces of oxygen in the system. The irregular nature of the effect suggested that the source of any oxygen was probably the muscle tissue rather than the argon used to obtain anaerobic conditions, but it was never-

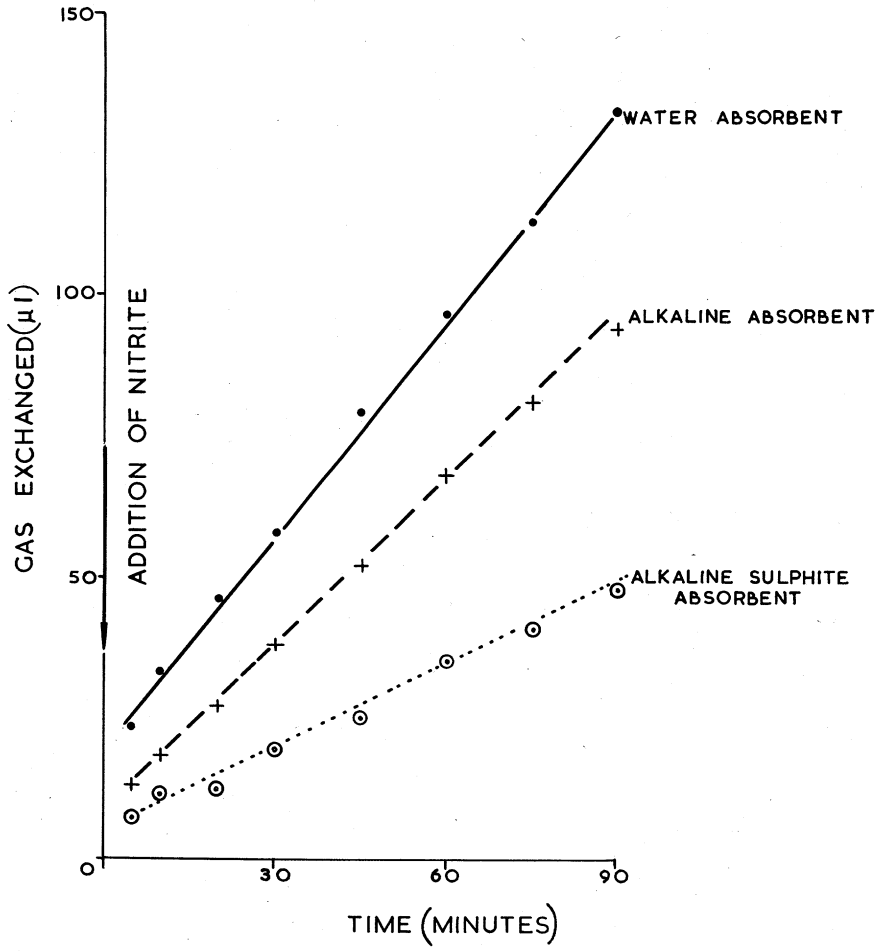

Fig. 1. Anaerobic gas evolution from pork muscle mince incubated at pH 6.0 with sodium nitrite.

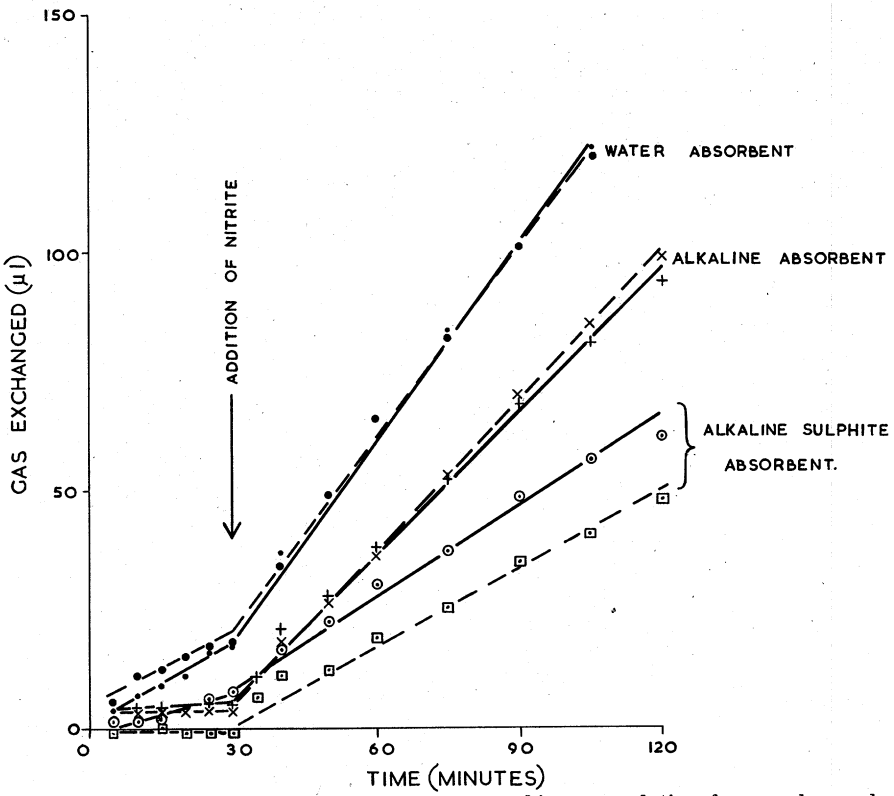

Fig. 2. Effect of addition of nitrite on anaerobic gas evolution from pork muscle mince at pH 6.0.

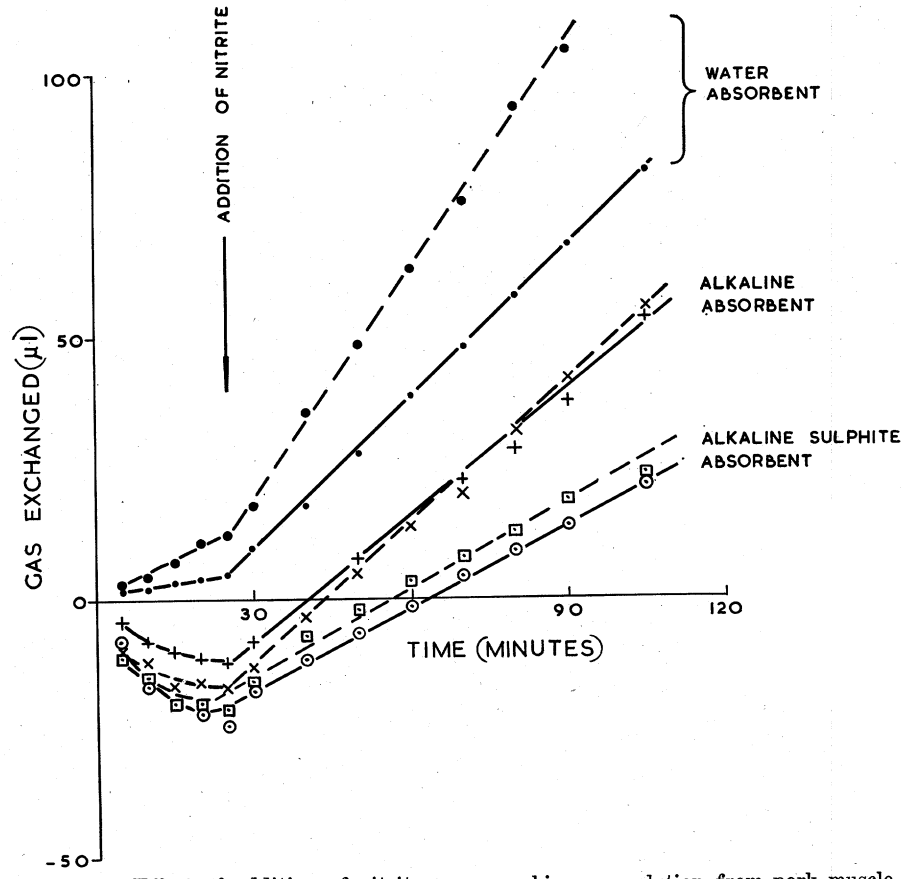

Fig. 3. Effect of addition of nitrite on anaerobic gas evolution from pork muscle mince at pH 6.0.

theless thought desirable to examine the effect of pre-treating the argon to eliminate any residual oxygen. Figs. 2 and 3 themselves show the results of this treatment; in both figures the full lines were obtained with untreated argon, and the broken lines were obtained with argon scrubbed in a 'Nilox' apparatus with a chromous salt solution claimed to reduce the residual oxygen content to 0.2 ppm. The absence of appreciable effect confirmed that the argon was not a significant source of oxygen. The initial absorption may still be due to traces of oxygen released from the tissues of the muscle mince. In any event, the immediate effect of the introduction of nitrite is again apparent in Fig. 3.

The presence of nitric oxide in the gaseous products has been confirmed by mass spectrometry and by infrared absorption spectrophotometry. Four trials were carried out with the mass spectrometer, using fresh minces prepared from a pork hock. In each trial a peak corresponding to a mass-charge ratio of 30 was observed and was ascribed to nitric oxide, the molar percentage ranging from 9 to 14. Nitrogen was present in all cases, and nitrogen dioxide in one. Peaks at a mass-charge ratio of 44 were observed in all trials, but these were ascribed to nitrous oxide rather than to carbon dioxide, owing to the absence of peaks at a mass-charge ratio of 22, corresponding to the loss of two electrons by carbon dioxide. Only 0·5% nitric oxide was found in a control trial without meat.

Carbon dioxide was identified by infrared absorption spectrophotometry. Nitric oxide could not be detected directly by this technique, owing to masking of its weak absorption at 1870 cm^{-1} by traces of water vapor remaining in the gaseous products even after drying. Admission of oxygen, however, resulted in a large increase in absorption at 1615 cm^{-1}, shown to be characteristic of nitrogen dioxide. No nitric oxide could be detected by this device in the gases from digestions either of buffer and sodium nitrite without mince or of buffer and mince without nitrite.

The enzymic character of the mechanism of production of presumptive nitric oxide is indicated by the fact that the activity of the muscle minces toward nitrite was quite sensitive to heat treatment. Table 1 shows the inhibition of activity in a number of minces heated at 56 and at 80°C.

The production of gas not absorbed by the alkaline sulfite reagent was

much less affected by the heat treatment, in accord with the suggestion that this gas was of chemical origin.

Incorporation of broad-spectrum antobiotics during the incubation of the mixture of nitrite and muscle mince did not affect the production of presumptive nitric oxide. Table 2 shows the results of two typical experiments with aureomycin and chloromycetin.

Conversely, enhancement of the bacterial population of the muscle mince by holding for 24 hr at 25°C led to a decrease in the production of presumptive nitric oxide, even when the activity of the organisms was not restrained by the addition of antibiotic during subsequent incubation with nitrite. Table 3 shows the growth of the bacterial population and the change in presumptive nitric oxide production after overnight holding in tests done both with and without the subsequent inclusion of antibiotic.

Observations of the gas exchanges from the minces after overnight holding were hampered by the initial absorption of carbon dioxide associated with the high metabolic activity of these tissues. The results in Table 3 nevertheless show that production of presumptive nitric oxide did not increase as bacterial count increased approximately 10,000-fold. The bacterial counts of all fresh minces were low and could have represented only slight metabolic activity. Moreover, the nitrite itself at the concentration used (approximately 2500 ppm overall) would have considerable bacteriostatic activity, particularly under anaerobic conditions (Castellani and Niven, 1955). It therefore seems justifiable to conclude that the production of presumptive nitric oxide was associated with mammalian rather than with bacterial enzyme systems.

The inclusion of antibiotic has been adopted as a routine precaution in subsequent gas exchange measurements. For this purpose, chloromycetin has been selected on account of its greater solubility, its broader spectrum of action, and the generally lower concentrations required for inhibition of bacterial growth (Baron, 1950).

The capacity to produce presumptive nitric oxide was maintained, although to a diminished extent, in the presence of sodium chloride in concentration similar to that encountered in normal curing operations. In two trials, carried out in the normal manner except for the inclusion of 10% (w/v) sodium chloride in the supporting phosphate buffer in some flasks, the average production of presumptive nitric oxide in the presence of salt was 0.28 and 0.30 μl/min, the figures for the corresponding salt-free controls being 0.34 and 0.42 μl/min.

As already stated in describing the experimental methods, observations were made at the normal temperature for Warburg manometry (37°C). Some experiments were subsequently made at 4°C to simulate conditions during commercial curing. At this temperature the differential manometric technique was too insensitive to detect the evolution of presumptive nitric oxide, even after 24 hr of incubation. A more sensitive technique, however, involving increases in optical density of the absorption liquids in the ultraviolet region, demonstrated the production of gaseous nitric oxide under these conditions.

Reduction of nitrate. Anaerobic incubation of fresh pork minces (capable of reacting with sodium nitrite) with potassium nitrate under the usual conditions gave no indication of the production of presumptive nitric oxide. Fig. 4 illustrates the lack of response of a typical incubation system toward nitrate, and the immediate effect of a later inclusion of nitrite. Even the more sensitive optical-density technique gave no consistent indication of the production of nitric oxide from fresh muscle minces incubated anaerobically with nitrate. Omission of the antibiotic did not affect the behavior of fresh minces under these conditions. When the bacterial count was enhanced by a preliminary holding period of 24 hr at 25°C, the minces showed

Table 1. Effect of heat treatment on production of presumptive nitric oxide by pork muscle mince (3 g muscle mince; 3.0 ml 0.2*M* phosphate buffer. pH 6.0, + 0.5 ml 3.0% w/v sodium nitrite solution; concentration of antibiotic in medium, 8.5 mg/100 g).

	Gas evolution (μl/min)					
	Before heat treatment			After heat treatment		
Heat treatment	Alkali absorbent	Alkaline sulfite absorbent	Difference (presumptive nitric oxide)	Alkali absorbent	Alkaline sulfite absorbent	Difference (presumptive nitric oxide)
10 min/56°C	0.45	0.19	0.26	0.25	0.16	0.09
	0.57	0.25	0.32	0.43	0.34	0.09
10 min/80°C	0.39	0.14	0.25	0.21	0.12	0.09
	0.50	0.24	0.26	0.12	0.11	0.01

Table 2. Effect of antibiotics on production of presumptive nitric oxide by pork muscle mince (3 g muscle mince; 3.0 ml 0.2*M* phosphate buffer, pH 6.0, + 0.5 ml 3.0% w/v sodium nitrite solution; concentration of antibiotic in medium 8.5 mg/100 g).

	Gas evolution (μl/min)					
	With antibiotic			Without antibiotic		
Antibiotic	Alkali absorbent	Alkaline sulfite absorbent	Difference (presumptive nitric oxide)	Alkali absorbent	Alkaline sulfite absorbent	Difference (presumptive nitric oxide)
Aureomycin	0.41	0.20	0.21	0.39	0.17	0.22
Chloromycetin	0.60	0.30	0.30	0.52	0.21	0.31

Table 3. Bacterial count and presumptive nitric oxide production of fresh minces and of minces held 24 hr at 25°C (3 g muscle mince; 3.0 ml 0.2*M* phosphate buffer, pH 6.0, + 0.5 ml 3.0% w/v sodium nitrite solution; bacterial counts on nutrient agar carried out after anaerobic incubation at 37°C in Warburg apparatus, generally for 90 min).

	Fresh mince		Mince held 24 hr at 25°C	
	Bacterial count (no./g)	Presumptive nitric oxide (μl/min)	Bacterial count (no./g)	Presumptive nitric oxide (μl/min)
Chloromycetin present (8.5 mg/100 g)	11.0×10^4	1.14	1.8×10^8	0.3
	4.0×10^4	0.40	1.2×10^8	nil
	6.0×10^4	0.30	2.0×10^8	nil
Chloromycetin absent	6.5×10^4	0.26	4.0×10^8	0.3
	2.0×10^4	0.71	2.5×10^8	0.5

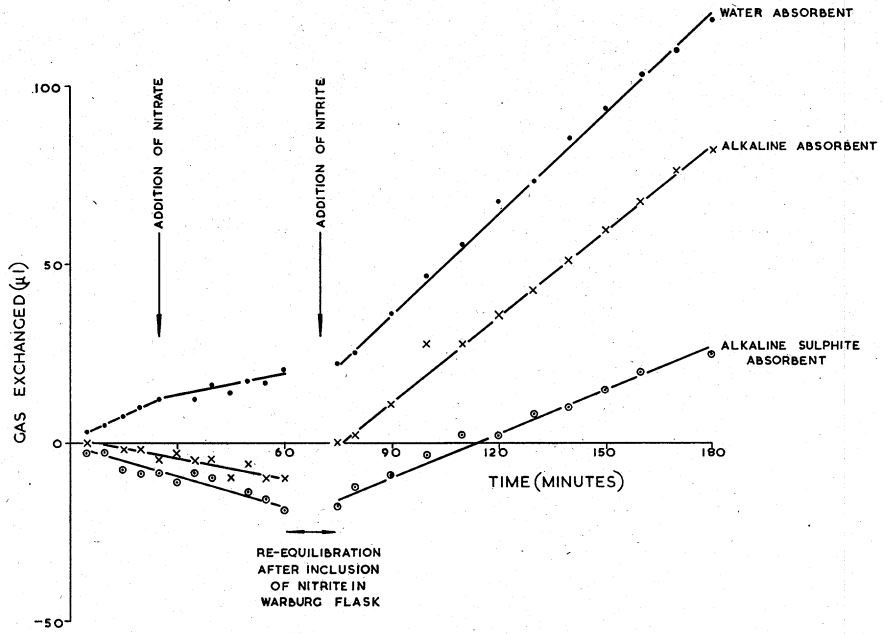

Fig. 4. Effect of addition of nitrate and nitrite on anaerobic gas evolution from pork muscle mince at pH 6.0.

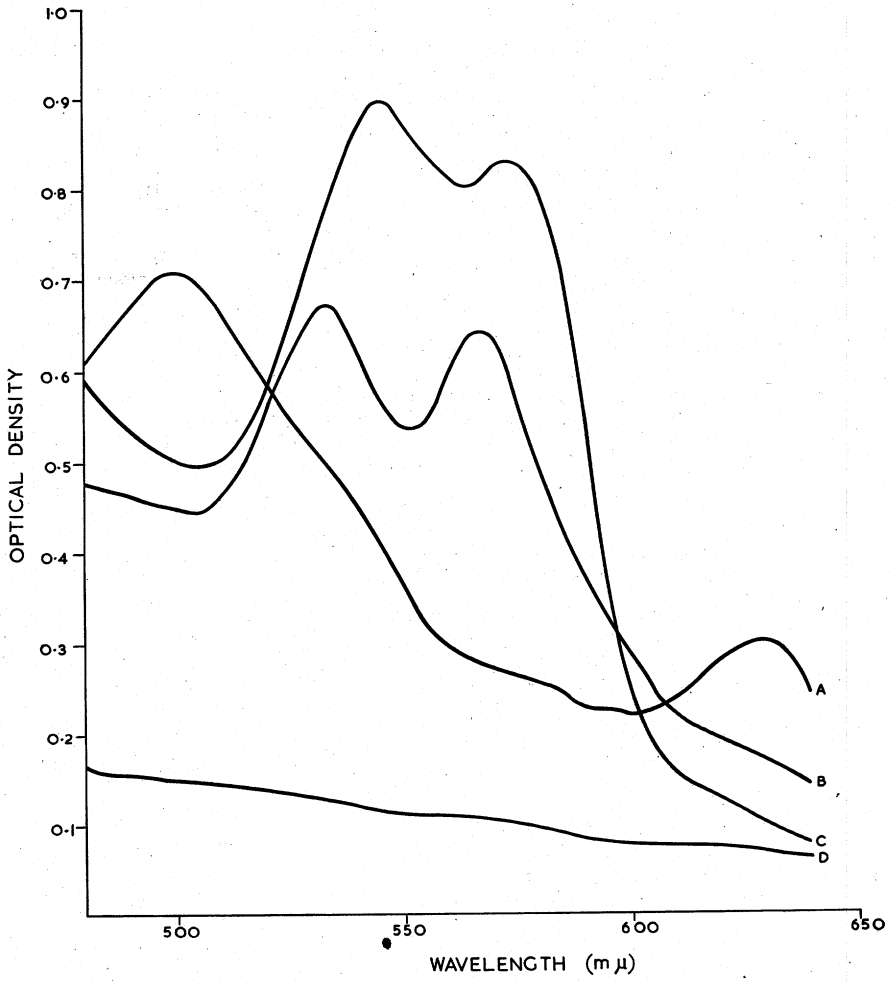

Fig. 5. Absorption spectra of pig heart myoglobin derivatives. A, pigment as isolated, metmyoglobin; B, nitric oxide-metmyoglobin complex; C, nitrosylmyoglobin; D, decomposition product of nitric oxide-metmyoglobin complex after exposure to air.

sporadic development of activity toward nitrate, even when chloromycetin was added during the subsequent Warburg incubation. Table 4 shows observations made in the absence of the antibiotic.

Table 4. Activity toward nitrate of minces with enhanced bacterial count (3 g muscle mince; 3.0 ml 0.2*M* phosphate buffer, pH 6.0 + 0.5 ml 3.0% w/v potassium nitrate; bacterial counts as for Table 2).

Fresh mince		Mince held 24 hr at 25°C	
Bacterial count (no. /g)	Presumptive nitric oxide (µl/min)	Bacterial count (no. /g)	Presumptive nitric oxide (µl/min)
2.1×10^4	nil	3.5×10^7	0.3
6.5×10^4	nil	4.0×10^8	0.2

The reduction of nitrate thus appears to be associated with a high bacterial count; there is no evidence of nitrate reduction by the mammalian enzyme systems.

Reduction of metmyoglobin. Table 5 shows the reduction of metmyoglobin on anaerobic incubation with fresh pork muscle minces in the presence of chloromycetin. It is clear that the minces were able to effect substantial reduction of the pigment in the presence of an inhibitory concentration of antibiotic. Further attempts to differentiate between the action of bacterial and mammalian enzyme systems in this context by repeating the experiments on muscle minces previously held 24 hr at 25°C to enhance their bacterial populations were not successful since satisfactory clarification of the products has not yet been possible with samples of high bacterial count.

Preparation and properties of nitric oxide-metmyoglobin complex. The freeze-dried pigment from pig hearts contained 0.32% iron, equivalent to one gram atom of metal per 17,500 g. The spectrophotometric absorption curve of this material in aqueous solution showed that it had become converted to the met form during the preparation, and that the product as isolated was in this form (see Fig. 5, Curve A). The pigment was soluble in 3*M* phosphate buffer of pH 6.8, and thus was essentially free of hemoglobin (George and Stratmann, 1952). Attempts failed to crystallize the pigment by dialysis of an aqueous solution at pH 6.8 in a Visking cellulose casing against saturated ammonium sulfate solution and by the maintenance of a solution in 36% (w/v) ammonium sulfate at 0°C for several days (Lawrie,

Table 5. Reduction of pig metmyoglobin by fresh pork muscle minces (2 g muscle mince; 6.0 ml water containing pig metmyoglobin [1.33 mg/ml] and chloromycetin [6.7 mg/100 g]; sodium iodoacetate added to 0.011*M* to arrest enzyme action).

Total pigment extracted (mg)	% of total pigment in met form; sodium iodoacetate added		Metmyoglobin reduced in 2 hr at 37°C (mg)
	Before incubation	After incubation	
12.3	65	43	2.7
12.4	65	43	2.7
12.8	57	50	0.9
10.7	100	73	2.9
7.5	79	36	3.3
10.7	**93**	**74**	**2.0**
9.7	100	76	2.3
7.1	70	14	4.0
6.3	74	24	3.1
10.1	74	60	1.4

1951); only an amorphous powder resulted. Lewis and Schweigert (1955) also failed to crystallize pork myoglobin, but, whereas they detected contaminating proteins by electrophoresis, in the present work examination of a solution of the pigment in phosphate buffer of pH 9.3 (ionic strength I = 0.5) in an Antweiler electrophoresis apparatus indicated one sharp symmetrical peak, anodic in migration.

Exposure of an aqueous solution of this met pigment to gaseous nitric oxide under anaerobic conditions resulted in a deep crimson color. The absorption spectrum of the solution (see Fig. 5, Curve B) exhibited maxima at 534 and 567 mμ, and showed no evidence of the original bands of the metmyoglobin. Fig. 5 also shows the absorption spectrum at pH 6.0 of the true nitrosylmyoglobin pigment, prepared by addition of sodium nitrite and dithionite to the met pigment in aqueous solution (see Fig. 5, Curve C). The absorption bands of the nitric oxide-metmyoglobin complex are displaced toward the blue end of the spectrum, in comparison with those of the true nitrosyl pigment (545 and 573 mμ) the respective shifts being almost exactly the same as those reported between the corresponding bands of the horse heart nitrosylmyoglobin and nitrosylmetmyoglobin of Ehrenberg and Szczepkowski (1960).

Displacement of excess nitric oxide by bubbling with argon had no effect on the nitric oxide-metmyoglobin complex, which was stable under argon at room temperature for at least 20 hr. Admission of air, however, resulted in rapid decomposition to a yellow-brown pigment having an absorption spectrum without pronounced peaks (see Fig. 5, Curve D). Light was not necessary for this decomposition. The brown end-product did not react again with nitric oxide to yield any red coloration, and no hemochrome formation with pyridine could be detected, suggesting that the heme moiety was no longer available. Judged by the persistence of the spectral bands (as observed in a Hartridge reversion spectroscope), the nitric oxide-metmyoglobin complex in aqueous solution under argon withstood heating for 20 min at 62°C, 10 min at 68°C, and 3 min at 74°C.

A similar red complex was produced between nitric oxide and pig methemoglobin (prepared by bubbling argon through a solution of pig oxyhemoglobin from hemolyzed pig erythrocytes); this complex absorbed maximally at about 535 and 570 mμ. On displacement of excess nitric oxide with argon, a stable red color was obtained with bands indistinguishable from those of pig nitrosylhemoglobin at 543 and 573 mμ; subsequent admission of air produced no further immediate change. This accords with the decomposition of the nitric oxide-methemoglobin complex (of unstated species) of Keilin and Hartree (1937), but contrasts sharply with the behavior of the corresponding myoglobin complex, which was quite stable under anaerobic conditions.

No evidence has so far been observed of the formation of this pigment *in vivo*, and its significance in relation to the general problem of color development and stability in curing has therefore still to be established.

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